Interaction of bioactive glasses with peritoneal macrophages and monocytes *in vitro*

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Abstract: Macrophage activation was analyzed following exposure to pure, crystalline α-quartz powders, two bioactive gel-glass powders of different compositions, and a melt-derived glass, 45S5 Bioglass®. The release of reactive oxygen metabolites (chemiluminescence test), modifications of cell morphology, the amount of tumor necrosis factor α (TNFα) secreted, and the amount of TNFα mRNA expression were evaluated. The 45S5 Bioglass® powders elicited the highest chemiluminescence response while the two sol-gel glasses had a lower response with less of an oxidative burst difference between them. Particulate bioactive glasses are actively ingested by mouse peritoneal macrophages, and only the 58S sol-gel glass had a moderate toxic effect on the macrophages. Macrophage cell morphology showed increased size and cell spreading, consistent with the high level of cytokine secretion induced by 45S5 Bioglass®. The 45S5 Bioglass® powders led to an increased release of TNFα and expression of TNFα mRNA relative to unstimulated and control treated monocytes. Bioactive glasses (and particularly 45S5 Bioglass®) that *in vivo* induce rapid bone growth appear to activate an autocrine-like process in which the response evoked by the material (for example monocyte and macrophage activation with cytokine production) enhances subsequent interactions with cells in contact with the material. © 2002 John Wiley & Sons, Inc. J Biomed Mater Res 60: 79–85, 2002

Key words: bioactive glass; macrophages; monocytes; TNFα; chemiluminescence

INTRODUCTION

The macrophage, a long-lived tissue cell derived from circulating monocytes, is a functionally advanced cell type involved in clotting, and the fibrinolytic complement cascade produces mediators that can induce the proliferation and protein synthesis of other cell types involved in inflammatory response and wound healing.

It has been shown that macrophages respond differently to bioactive and bioinert materials.[1] Bioactive glasses are a class of biomaterials the reactivity of which depends on the dissolution of surface ions and rapid formation of a silica-rich hydroxycarbonate apatite (HCA) layer on the glass surface to which bone can bind with a mechanically strong interface.[2] With the sol-gel process, a new generation of bioactive materials that enhance the regeneration of natural tissues has been achieved.[2] Bioactive gel-glasses have a large surface area rich in chemically reactive silanols. Thus bioactive gel-glasses nucleate a biologically active HCA layer within minutes, a rate that is significantly more rapid than that of bioactive melt-derived glasses.[3] Sol-gel processing also makes it possible to obtain a much wider range of silica content and variable levels of CaO and P_2O_5 content.[2,3] Thus gel-glass compositions with as much as 77% SiO_2 (77S) have a rate of bone formation *in vivo* equivalent to 45S5 Bioglass® (45S5), a melt-derived glass with 45% SiO_2 weight percent. Compositions of gel-glasses in the range of 58% SiO_2 (58S) have even higher rates of resorption and bone formation.[4] These differences are attributed to a more rapid release of soluble silica that accelerates the heterogeneous nucleation of HCA crystals in the very small pores of the gel-glass[5] and also influences the osteoblast cell cycle[6] and expression of genes by osteoprogenitor cells.[7,8] These recent studies show that the slow release of critical concentrations of soluble silica has a large influence on the biologic response to bioactive glasses of differing compositions, surface areas, and dissolution rates.[6–8]

It is well known that in pulmonary fibrosis there is an activation of numerous cells (macrophages, lymphocytes, and neutrophils) by SiO_2 that produces cy-

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tokines that regulate the proliferation, chemotactism, and secretory activity of fibroblasts.[9-11] Consequently, to further understand the relative importance of the surface activity and dissolution of SiO_2-containing particulate, we have evaluated the effect of dense melt-derived bioactive glass particles (45S5 Bioglass®) versus porous sol-gel-derived SiO2-based gel-glass particulate on the cell activity of peritoneal macrophages and blood monocytes. Pure crystalline SiO_2 (α-quartz) powders were used as a control as were cell cultures with no powders added.

MATERIALS AND METHODS

Materials

Respiratory burst measurement and cell morphology studies were made of four materials of different composition:

1. Crystalline silica (α-quartz) commercial powder 0.5–10 μm in diameter (Sigma, Milan, Italy) was used as a reference material.
2. Bioactive glass, 45S5 Bioglass® (46.1% SiO_2, 24.4% Na_2O, 26.9% CaO, 2.6% P_2O_5, all in mol %). The glass was prepared by melting reagent-grade chemicals at 1325°C in a covered platinum rhodium crucible, homogenizing them for 24 h, casting, crushing, and sieving them to 90–700-μm particles (the powders were obtained from U.S. Biomaterials Corp., Alachua, FL).
3. and 4.Bioactive gel-glass powders 58S and 77S (100–700 μm in diameter) were made from tetraethylorthosilicate, triethylphosphate, and calcium alkoxide using the sol-gel process previously described.[3] After mixing the alkoxide components, the sol was cast into polyethylene containers, loosely covered, and placed inside a desiccator containing water. Hydrolysis of the alkoxides occurred with moisture from the ambient atmosphere, and gelation occurred within 3 days. The gel was aged at 60°C and dried with a schedule ending at 180°C. The dried gels were heated in air at 700°C and the textural features measured as described in a previous paper.[3] The nominal composition for the 58S was 60% SiO_2, 36% CaO, 4% P_2O_5 (all in mol %); and for 77S it was 80% SiO_2, 16% CaO, 4% P_2O_5 (all in mol %). All materials were sterilized in dry heat at 180°C for 3 h before use.

Peripheral human blood mononuclear cells (MC) isolation

Heparinized peripheral human blood from healthy volunteers was centrifuged in Cellsep™ monocytes gradient (Larex, Inc., St. Paul, MN) at 1000 g for 20 min at 20°C. After two washes at room temperature in Hanks' Balanced Salt Solution (HBSS; Sigma), the isolated cells were resuspended at a density of 2.5×10^5 cells/mL in minimal essential medium (MEM) containing 10% heat-inactivated fetal calf serum, 2 mM of glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL), all from Sigma. Trypan blue staining and counting in a hematocytometer determined cell viability and number.

Peritoneal macrophages (PM) isolation

Peritoneal macrophages were collected by flushing the peritoneal cavities of Balb C mice with approximately 8 mL of cold PBS. The pooled fluids were centrifuged for 10 min at 1000 g in polypropylene tubes (Corning Glass Works, Corning, NY). Then the cells were suspended in RPMI 1640 culture medium (GIBCO, Grand Island, NY) containing 20% heat-inactivated fetal bovine serum (FBS; 10 μL/mL); glutamine (200 mM; 10 μL/mL); streptomycin/penicillin G solution (10 μL/mL; and fungizone® (250 μg/mL). Routinely 4×10^6 cells were harvested from each mouse and approximately 1×10^6 cells adhered to the plate.

Cell culture and cell–material interactions

The PMs were plated at 4×10^6 cells/well in a 6-well multidish plate (Corning). After overnight incubation in a CO_2 incubator (5% CO_2 in air) at 37°C, nonadherent cells were removed by washing with phosphate-buffered saline (PBS), and fresh medium containing antibiotics, glutamine, and 5% FBS with or without particulate was added to each well. Cell stimulation was obtained by adding 5 mg of materials to test in the form of powders. The PMs also were cultured without any stimulation in control wells. The PM cells were allowed to incubate for 3 days at 37°C in a 95% air/5% CO_2 atmosphere, and the supernatants from all cultures were harvested, centrifuged for 3 min at 14,000 g, and aliquots of the cell-free cultured media were taken for TNFα measurement in culture medium. Adhered cells were treated to extract total cellular RNA to study TNFα mRNA expression.

Measurement of respiratory burst

Blood mononuclear cell activation was determined by chemiluminescence assay (CL) using 1,000,000 cells suspended in 3 mL of PBS (37°C) in propylene vials (Packard Company, Milan, Italy). The CL was monitored in a Beckman CPM-100 liquid scintillation counter normalized and programmed in the single photon counting mode with 1-min counts per well and ten counting cycles. Luminol (Sigma) was added to the cell suspensions to give a final concentration of 0.1 mM. Subsequently, CL was elicited by addition of 400 μg/mL of the powders to test. Cells with luminol were used as negative controls. Results are the average of nine experiments, and statistical evaluation was made using an independent sample t test. P values were

obtained from the ANOVA table and the conventional 0.05 level was considered to reflect statistical significance.

Cell morphology

Peritoneal macrophages (PM), after rinsing in PBS, were fixed for 20 min at 60°C and stained with a 0.025% (weight/volume, w/v) acridine orange solution, a nucleic acid staining dye. Cell–material interactions were analyzed using a fluorescence microscope (Aristoplan, Leitz, Milan, Italy).

TNFα immunoblotting

To evaluate PM TNFα production, culture media were dialyzed against distilled water for 24 h, freeze-dried, and resuspended in 20 μL of SDS-PAGE sample buffer [2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol, and 0.02% (w/v) bromophenol blue in 62.5 mM of Tris-HCl, pH 6.8]. The samples (15 μL with 3 μg of protein) were loaded onto 10% SDS-PAGE, and electrophoresis was carried out at 100 V by a Mini-Protein II electrophoresis system (Bio-Rad, Milan, Italy). Proteins were stained by a Bio-Rad Silver Stain Plus kit or blotted onto nitrocellulose membranes (Amersham, Milan, Italy) by Burnette's method in a Mini Trans-Blot electrophoresis Transfer Cell (Bio-Rad). The membranes were treated with 1% (w/v) gelatin in phosphate-buffered saline (PBS; pH 7.4) (0.01*M* of phosphate buffer, 0.0027*M* of KCl, 0.137*M* of NaCl) for 1 h at room temperature and then incubated with 50 μg/mL of primary antibody solution overnight at 4°C. The monoclonal antibody used in this study was provided by Dr. Bellone. After washing procedures, the membrane was incubated with 1:2000 diluted secondary antibody conjugated with horseradish peroxidase (Amersham) for 1 h at room temperature. Primary and secondary antibody dilutions were carried out in 0.1% (w/v) gelatin in PBS. The same solution also was used for washing procedures. ECL™ Western blotting detection reagents (Amersham) were used for immunodetection of the eluted proteins. The amount of TNFα secreted was studied on the reference material (SiO_2 α-quartz) and on 4555 Bioglass® as the highest macrophage activator from the preliminary studies.

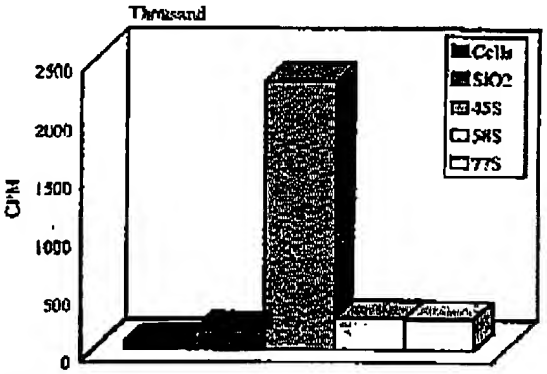
TNFα RT-PCR

Total cellular RNA was extracted using a Qiagen QIAshredder and RNeasy kit (Qiagen, Germany). Complementary DNA (cDNA) was prepared from 2 μg of total RNA using random primers (2.5 μ*M*; Gibco-Life Technologies, Milan, Italy) and sterilized water to adjust the final volume to ~20 μL. The reaction mixture was heated at 60°C for 5 min. $MgCl_2$ (50 m*M*o) 2-μL buffer 10× (Gibco); 2 μL of dNTP10 m*M* (Gibco); 0.25 U of ribonuclease inhibitor (Aldrich, Milan, Italy), and 1 μL of M-MLV transcriptase (Gibco) then were added. The reaction mixture was heated at 42°C for 1 h to obtain cDNA and then was heated at 95°C for 1 min to eliminate RNA. Before PCR treatment, the sample was centrifuged at full speed (14,000 rpm or more). The synthesized cDNA then was used for the polymerase chain reaction (PCR). This technique exponentially amplifies nucleic acid sequences. The PCR primers for TNFα were as follows: 5′- GAAAGCATGATCCGCGACGT –3′ and 5′-AGACCTGCCCGGACTCCG-3′ (TIB MOLBIOL, Genoa, Italy). Amplification was performed in a DNA Thermal Cycler (PerkinElmer Cetus). Thermocycling conditions for TNFα were an initial denaturation step at 95°C for 1 min followed by 30 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 60 s. Reaction product was analyzed by electrophoresis of a 10-μL sample in 2 % agarose gel to which ethidium bromide had been added (1 μL of 10 mg/mL in 100-mL gel solution). The gels were analyzed under ultraviolet light at 302 nm using a Gel Doc 1000 software Quantity One (Bio-Rad) TNFα mRNA expression was studied on the reference material (SiO_2 quartz) and on 4555 as the highest MC and PM activator from the preliminary studies.

RESULTS

Respiratory burst

For all materials a chemiluminescence (CL) response by the mononuclear cells was detected (Fig. 1). All four glasses evidenced an increase of the CL re-



	Peak oxidative burst (cpm), Mean± SD n=9
Cells	73483 ± 40806
SiO2	177197 ± 70621
45S*°	2314708 ± 1027660
58S*	278613 ± 126855
77S*	274906 ± 200755

* $p < 0,05$ respect to control cells
° $p < 0,05$ respect to 58S; 77S; SiO2

Figure 1. Materials induced chemiluminescence response by human peripheral blood monocytes/macrophages. The results are based on mean values of nine measurements.

sponse relative to the control (unstimulated) cells ($p < 0.05$). The 45S5 powder induced the highest CL response and resulted in a statistically higher response ($p < 0.05$) with respect to 58S and 77S bioactive gel-glass powders.

Cell morphology

The overall shape and the presence of pseudopodia were observed for the peritoneal macrophage cultures. Figure 2(a) shows PMs cultured without any stimulation in the six-well multidish plate (polystyrene) where cells evidenced their round shape. Figure 2(b) shows, at the same magnification, PMs grown in the presence of SiO_2 quartz; cells that revealed a higher dimension with round-shape morphology actively ingested the particles. The 45S5 Bioglass® stimulation [Fig. 2(c)] evidenced a very different cell shape from the control; for example, the PMs adhered and most of them spread extensively. The 58S gel-glass stimulation [Fig. 2(d)] resulted in the cell density being very low and the cells showing some features that indicated spreading (filopodia). Cell behavior in the presence of the 77S gel-glass powder appeared similar to the control PM cultures, maintaining mostly a rounded shape and features typical of healthy cells [Fig. 2(e)].

TNFα production

The results of the immunoblotting for TNFα after 3 days of exposure to the various biomaterials are shown in Figure 3. TNFα production significantly was augmented in the presence of 45S5 powders (lane 2) and SiO_2 quartz (lane 3) compared to the control (lane 1). The greatest increase was seen in the presence of 45S5 Bioglass® powders.

TNFα-mRNA

Ethidium-bromide-stained agarose gel of PCR (Fig. 4) demonstrated an increase in PM mRNA encoded by the gene for TNFα when PMs were incubated in the presence of 45S5 Bioglass® powders (lane 2) relative to control cells (lane 4) and SiO_2-quartz-stimulated cells (lane 3). Synthesized DNA fragments were predicted from the published nucleotide sequence and the oligonucleotide primers used, that is, 708 nucleotides for TNFα.

DISCUSSION

Bioactive glasses have shown promising results as bone-graft augmentation materials that lead to accelerated rates of bone formation in osseous defects[12,13] and are extensively used clinically.[14,15]

Macrophage activation by biomaterials is a widely accepted mechanism involved in host biocompatibility response.[16] It is known that cell–biomaterial contact evokes the release of chemotactic mediators and growth factors that may elicit and sustain inflammatory responses at the implant site.[17] Several studies have shown that macrophages respond to a material with cytokine release,[18] size and morphology changes,[19] and phagocytosis with subsequent release of oxygen metabolites.[20]

Silicon dioxide, in the form of crystalline quartz, has been studied extensively to understand the development of pulmonary fibrosis.[11] Silica induces free radical production and respiratory burst in alveolar macrophages,[21] is cytotoxic and genotoxic to alveolar macrophages, which is related to its physicochemical properties, especially to its very high negative surface charge at physiological pH, and the effects are mediated by oxidative stress and reactive oxygen species formation.[22,23] This leads to an increase in lung macrophage expression of intercellular adhesion molecule-1 (ICAM-1) mediated by TNFα and oxygen species.[24]

Since bioactive glasses are surface-active implant materials, some type of surface activity is needed to achieve a successful implant material. On the other hand, it is known that materials that elicit a strong chemiluminescence response lead to an increase in oxygen uptake, production of $O_2^{\cdot -}$, OH^-, H_2O_2, and hexose monophosphate shunt activity, which have been associated with a variety of cell reactions in the surrounding tissue and with many diseases, such as chronic fibrotic lung disease and malignancies.[11,21–24]

In this study, the effects of three different bioactive glasses and gel-glasses on PM and MC were studied *in vitro*. Comparing human unstimulated MC relative to glass-treated cells, all glass-based materials evidenced a statistically significant increase in CL values, an index of increased cell release of reactive oxygen metabolites. The melt-derived glass 45S5 elicited the highest CL response, followed by the two sol-gel glasses, which evidenced no oxidative burst difference between them.

Crystalline SiO_2 powders and particulate bioactive glasses actively are ingested by PM. Only the 58S gel-glass powders seemed to have a toxic effect on them. The cell morphology study, in fact, evidenced a lower cell number in the presence of 58S after 3 days of incubation with some lysate cells and plasma membrane residues. The highest CL activator, 45S5, evidenced its activity also on PM cell morphology, which showed increased size and cell spreading that is correlated with macrophage activation[19] and is consistent

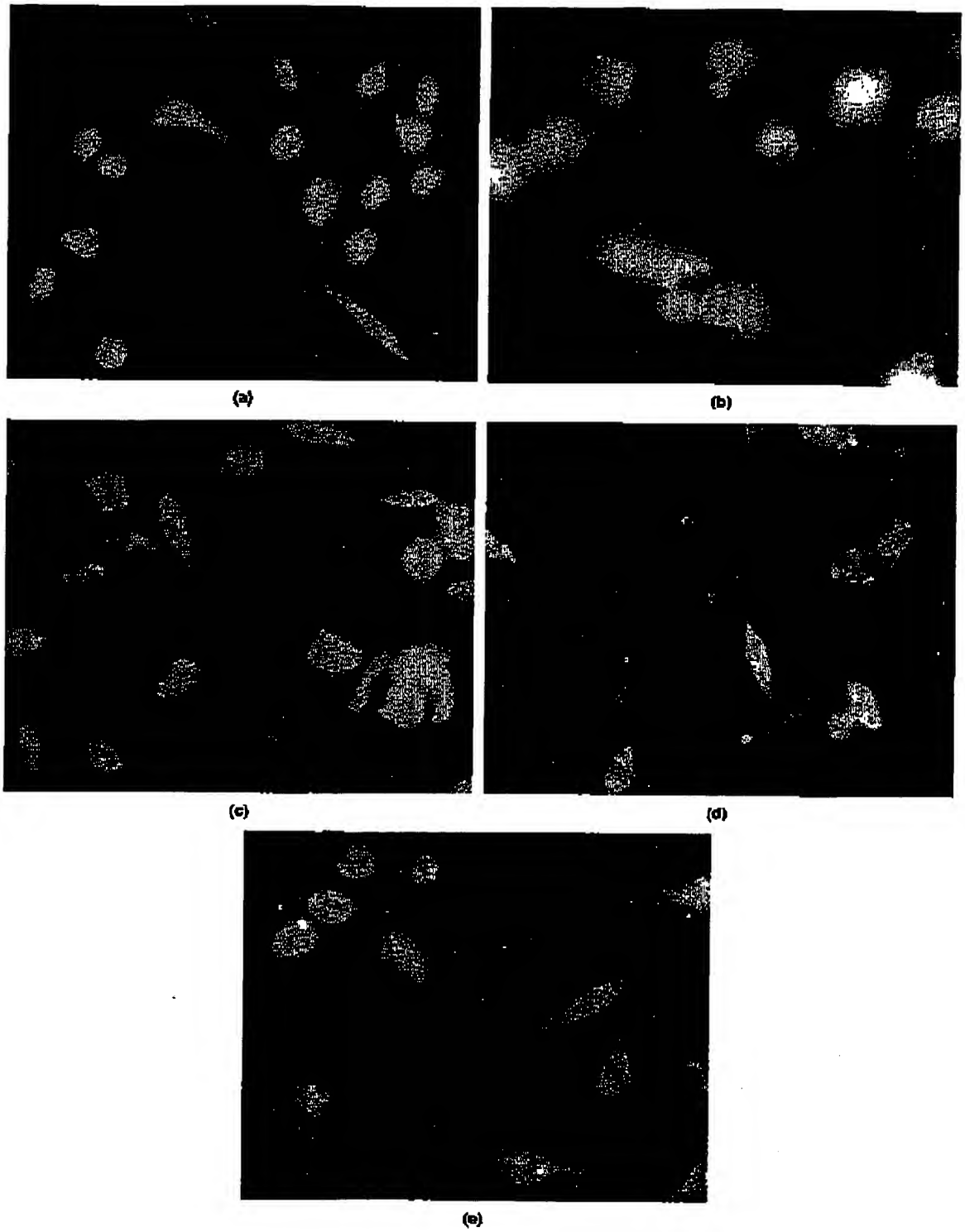


Figure 2. Mouse peritoneal macrophages stained with acridine orange solution to evidence green epifluorescence when bound to DNA and orange epifluorescence when bound to RNA (original magnification ×400): (a) control unstimulated cells; (b) cells incubated with SiO_2 crystalline powder; (c) cells incubated with 45S5 Bioglass; (d) cells incubated with 58S5 sol-gel glass; and (e) cells incubated with 77S sol-gel glass.

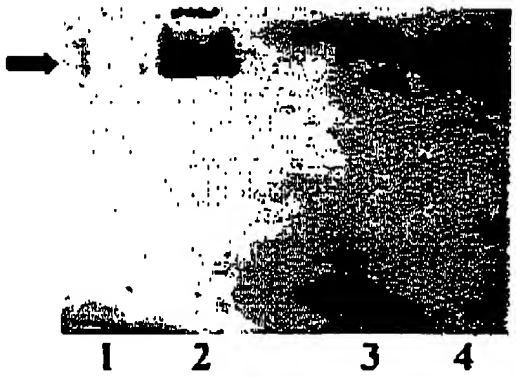


Figure 3. Immunoblot of TNFα using a monoclonal mouse anti-human TNFα antibody followed by horseradish peroxidase conjugated antibody and chemiluminescence (ECL) western blotting detection reagent. Lane 1: control unstimulated macrophages; lane 2: macrophages cultured in presence of 45S5 Bioglass; lane 3: macrophages cultured in presence of control material SiO_2 (quartz).

with the high level of cytokine secretion observed in our study. We have examined *in vitro* TNFα production (a marker for cytokine production) by PM cultured for 3 days in contact with SiO_2 quartz and 45S5 Bioglass®.

The results of TNFα release paralleled other *in vitro* studies that showed no relationship between particle phagocytosis and TNFα release.[25] The SiO_2 quartz particles that evidenced high PM phagocytosis [Fig. 2(b)] showed no macrophage TNFα release while 45S5, which was not highly phagocytosed, evidenced an increased TNFα release relative to untreated cells. Moreover, it is known that TNFα is synthesized *de novo* in stimulated macrophages and not stored intracellularly,[26] supporting its secretion by particle stimulation rather than by liberation of intracellular stores. In fact, 45S5 showed in RT-PCR analysis an increased expression of TNFα mRNAs relative to unstimulated and SiO_2-quartz-treated cells.

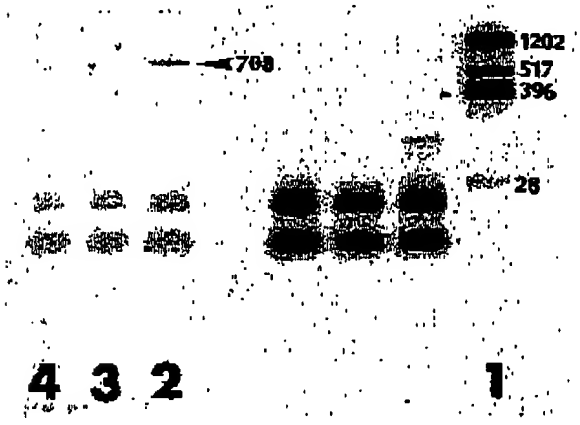


Figure 4. Ethidium-bromide-stained 2% agarose gel of polymerase chain reaction (PCR) generated cDNA fragment for TNFα. Lane 1: HindII cut pBluescript®; lane 2: macrophages cultured in presence of 45S5 Bioglass (arrow); lane 3: macrophages cultured in presence of control material SiO_2 (quartz); lane 4: control unstimulated macrophages.

In summary, 45S5 is a surface-active implant material with an effect on MC and PM, and the induction of increased expression of TNFα does not require phagocytosis. This result suggests that cellular contact with orthopedic implants is sufficient to induce cell activation and the release of cytokines that may determine prostheses survivability.

The complex mixture of cytokines that are produced by macrophages during inflammation may influence the behavior of the other cells that are recruited to the biomaterial implant. For example, Miller and Anderson[1] found that human peripheral blood monocytes cultured in the presence of several materials release factors that stimulated fibroblast proliferation and collagen production.

We have considered TNFα as a representative cytokine produced by macrophages in response to crystalline SiO_2 quartz and 45S5 Bioglass®, but other cytokines may act to enhance subsequent interactions of biomaterials with tissues. TNFα has regulatory effects on phagocytic cells, the enhanced production of oxidative radicals, increased degranulation, increased receptor expression, and increased eosinophils for parasitic infections.[27,28] The role of TNFα on osteoblast function is not very clear although it seems to have an indirect role through IL6 in the pathogenesis of periprosthetic osteolysis through a reduced periprosthetic bone formation due to inhibition of osteoblast proliferation, alkaline phosphatase production, and osteoclastogenesis.[29–31]

CONCLUSIONS

Bioactive glasses (and particularly 45S5 Bioglass®) that *in vivo* induce rapid bone growth appear to activate an autocrine-like process in which the response evoked by the material, for example monocyte and macrophage activation with cytokine production, enhances subsequent interactions with cells in contact with the material.

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